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(54) Titre : ANALOGUES DE NUCLEOTIDE PURIQUE SUBSTITUES EN POSITION C8 ET LEUR UTILISATION
COMME INHIBITEURS DES NUCLEOSIDE-TRIPHOSPHATE-DIPHOSPHOHYDROLASES

(54) Title: C8-SUBSTITUTED PURINE NUCLEOTIDE ANALOGS AND THEIR USE AS INHIBITORS OF NUCLEOSIDE
TRIPHOSPHATE DIPHOSPHOHYDROLASES

Substrates		K _m (μM)	V _{max} (μmol/min/mg protein)	Inhibitors		K _i (μM)
ATP		18 ± 1	1.65 ± 0.10	8-cycloheptylS-ATP	6a	31 ± 2.5
ADP		33 ± 1	1.30 ± 0.08	8-CH ₂ BuS-ATP	6b	45 ± 2.5
2-BuS-ATP	2a	36 ± 6	0.83 ± 0.05	8-hexylS-ATP	6d	16 ± 2.0
2-BuS-ADP	2b	63 ± 14	0.94 ± 0.10	8-BuS-ATP	6e	10 ± 2.0
2-BuNH-ATP	2c	32 ± 8	0.99 ± 0.10			
2-BuO-ATP2d	2d	28 ± 8	0.82 ± 0.09			
8-bromo-ATP		22 ± 5	0.63 ± 0.04			
8-ethylS-ATP	6c	12 ± 5	0.30 ± 0.03			
8-BuNH-ATP	7	20 ± 7	0.28 ± 0.03			
8-BuO-ATP	8	26 ± 5	0.20 ± 0.01			

(57) Abrégé/Abstract:

Ectonucleoside triphosphate diphosphohydrolases [NTPDases; EC 3.6.1.5] constitute a family of enzymes which are involved in the metabolism of extracellular nucleotides, catalysing the hydrolysis of the gamma and beta phosphate bonds of triphospho- and diphosphonucleosides (whereas 5'nucleotidases [EC 3.1.3.5] catalyse the hydrolysis of alpha phosphate bond of monophosphonucleosides). These extracellular nucleotides interact with endothelial, epithelial and smooth muscle cells, as well as blood cells and lymphoid cells, to influence the different physiological systems of vertebrates. Since these ecto-nucleotidases alter the extracellular concentrations of nucleotides these enzymes modulate their physiological effects, including, for example, platelet aggregation, heart function, control of vascular tone and inflammation reactions, electrolyte secretion and gastrointestinal motility, neurotransmission both in central and peripheral nervous systems, as well as other effects in other physiological systems. This invention provides C8 substituted purine nucleotide analogues, such as ATP analogues, and further provides their use as inhibitors of NTPDases and thereby as tools to modulate the conversion of nucleotides into nucleoside derivatives, and thus modulate the levels of these compounds. Such modulation further provides for the modulation of the activity and function of many processes which are affected by these compounds.

ABSTRACT

Ectonucleoside triphosphate diphosphohydrolases

[NTPDases; EC 3.6.1.5] constitute a family of enzymes which are involved in the metabolism of extracellular nucleotides,

5 catalysing the hydrolysis of the gamma and beta phosphate bonds of triphospho- and diphosphonucleosides (whereas

5'nucleotidases [EC 3.1.3.5] catalyse the hydrolysis of alpha phosphate bond of monophosphonucleosides). These extracellular

nucleotides interact with endothelial, epithelial and smooth
10 muscle cells, as well as blood cells and lymphoid cells, to influence the different physiological systems of vertebrates.

Since these ecto-nucleotidases alter the extracellular concentrations of nucleotides these enzymes modulate their physiological effects, including, for example, platelet

15 aggregation, heart function, control of vascular tone and inflammation reactions, electrolyte secretion and gastrointestinal motility, neurotransmission both in central and peripheral nervous systems, as well as other effects in other physiological systems. This invention provides C8

20 substituted purine nucleotide analogues, such as ATP analogues, and further provides their use as inhibitors of NTPDases and thereby as tools to modulate the conversion of nucleotides into nucleoside derivatives, and thus modulate the levels of these compounds. Such modulation further provides for the modulation
25 of the activity and function of many processes which are affected by these compounds.

**C8-SUBSTITUTED PURINE NUCLEOTIDE ANALOGS AND THEIR USE AS
INHIBITORS OF NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASES**

FIELD OF THE INVENTION

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The present invention relates to C8-substituted purine nucleotide analogs and their use as inhibitors of nucleoside triphosphate diphosphohydrolases (NTPDases), and is particularly concerned with such compounds which provide effective and specific inhibition of NTPDases.

BACKGROUND OF THE INVENTION

In 1971, results of extensive studies on neurotransmission, which was resistant to conventional adrenergic and cholinergic antagonists, led Burnstock to propose that the purine nucleotide ATP and/or the purine nucleoside adenosine, released at synaptic junctions, might mediate a non-adrenergic, non-cholinergic signalling (1-7). Burnstock also hypothesized that nerves released purines which interact on their target cells with purinergic receptors (or purinoceptors) for either ATP, or its breakdown product adenosine (8, 9). The putative ATP-selective receptors were termed P₂-purinoceptors, whereas the adenosine receptors, were termed P₁-purinoceptors (10). Soon, purinoceptors were identified, characterized, and localized in a variety of systems, organs, cells and cell extracts. At the beginning, purinoceptors were classified according to their pharmacological and physiological properties, but with the advent of molecular biology tools, genes encoding purinoreceptors were cloned and a new classification emerged (see 11 for a complete review). Extracellular ATP and ADP and its metabolite adenosine exert multiple effects through these purinoceptors. In the cardiovascular system, these compounds

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influence platelet aggregation, vascular tone, heart function and recruitment of blood cells involved in inflammatory processes (12-15). In the digestive system, it affects electrolyte secretion, gastrointestinal motility, stomach acid secretion and other secretions coming from accessory glands: parotid, liver and exocrine pancreas (16-20). Presence of purinoceptors in the immune system also support a role of extracellular purines and pyrimidines in the immune response (11, 21-25). Presence of these receptors in the central and peripheral nervous systems also supports a role in neurotransmission for these compounds (26-29). These localizations combined with the effects induced by the administration of nucleotides confirm the functions of these nucleotides and their metabolites.

15 A fundamental question is what determines extracellular concentrations of nucleosides and nucleotides in the extracellular compartment. Basically, there are five parameters involved: 1-Rate of release from the source (cell); 2-Rate of diffusion and size of the extracellular compartment; 20 3-Metabolism by ectonucleotidases; 4-Binding to proteins on the cell surface; and 5-Uptake by the cells (translocation or endocytosis). Ectoenzymes with ectonucleotidase activities often localized in proximity of the target cells are believed to play key roles as modulators of the purine or pyrimidine 25 actions. Among the ectoenzymes which display ectonucleotidase activities, one finds alkaline phosphatase [EC 3.6.1.3] which is widely distributed in the different systems of the body, protein kinase reported in certain cell types, ecto-nucleotide pyrophosphatase/phosphohydrolase [EC n.d.] which converts 30 nucleoside triphosphate into nucleoside monophosphate and 5'-nucleotidase [EC 3.1.3.5] which convert nucleoside monophosphate into nucleoside (30-34).

Ectonucleotidases, often located on the target cells, rapidly dephosphorylate the nucleotide into the corresponding nucleoside thereby ending the P_2 stimulation and thereby inducing a P_1 type stimulation (31, 33, 36). Quite often, the physiological response elicited by the nucleoside antagonizes the action induced by the corresponding nucleotide (adenosine vs ATP) (14-15). Adenosine is generally considered as a negative feedback modulator (retaliatory metabolite) of cell and organ energy demand and consumption. It interacts with P_1 purinoceptors which comprise at least four subtypes A_1 , A_{2A} , A_{2B} and A_3 , first classified into those that inhibit (A_1) and those that stimulate adenylate cyclase (A_2) (11). They were later classified according to their pharmacological properties and they are now distinguished by their amino-acid sequences (11).

Once released, nucleotides and nucleosides diffuse in the extracellular space and reach their receptor on target cells. Many enzymes contribute to the extracellular metabolism of nucleotides including alkaline phosphatase, ectokinases and deaminases. Perhaps the most important ones are those that convert nucleotides into nucleosides. Many reports have described ecto-ATPase, ecto-ADPase, and ecto-5'-nucleotidase activities in a variety of tissues and cells. The latter was purified, characterized biochemically, and its encoding gene was defined (34). As for the conversion of ATP to ADP and AMP, up until recently, it was believed that two distinct ecto-enzymes were involved in the conversion of ATP to ADP, and ADP to AMP, i.e., ecto-ATPase and ecto-ADPase, respectively (30). The detection of the NTPDase at the surface of vascular cells has presented another alternative for the conversion of ATP to AMP at the cell surface (37). The identification of a mammalian ATP diphosphohydrolase or apyrase goes back to the early 1980s when LeBel et al. described an enzyme that could sequentially catalyse the hydrolysis of γ and β phosphate residues of triphospho- and diphosphonucleosides (38). In a

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series of studies, the enzyme was purified, characterized, and identified as an ectoenzyme (39). A second isoform was identified, purified, and characterized in the bovine aorta (40) and placenta (41). Recent reports describing the homology between potato apyrase and human CD39, showing a comparison of bovine and porcine ATPDases, and the cloning and sequencing of the human ATPDase cDNA and reexpression of the human protein in COS cells, led to the demonstration that ATPDase isoform II and CD39 were the same protein (42-44).

Among many reported inhibitors of NTPDases, one finds analogs of purines, heavy metals, such as Cd^{2+} and Hg^{2+} (44, 46) and molecules belonging to the suramin family, Evans blue and also other types of molecules.

Purine analogs, such as β , γ -MetATP, β , γ -imido-ATP and ADP β S, may be used to inhibit the NTPDase (47). These analogs share a common characteristic, that is they all bear a substituted group on the phosphate chain. Moreover, all these analogs are purinoceptor ligands. Other nucleotide analogs have also been reported as NTPDase inhibitors, mainly ARL67156 and PPADS. These analogs have been reported to inhibit ecto-ATPase activity (48-51). Finally, two other purine analogs have been reported as NTPDase inhibitors: fluorosulfonylbenzoyl adenosine (FSBA) and 2-thioether-AMP-S (46, 52). However, contrary to purine analogs, FSBA causes an irreversible NTPDase inhibition.

Many P2 antagonists related to suramin (53), reactive blue (54), reactive red (55), Evans blue (56), trypan blue (56) and small aromatic isothiocyano-sulphonates (57), have been reported to be ecto-nucleotidase inhibitors. Other molecules have been proposed as non-specific NTPDase inhibitors, such as sodium azide, sodium fluoride (46) and 9-amino-1, 2, 3, 4-tetrahydroacridine or THA (58).

Based on the facts that (a) NTPDases play a major role in the regulation of purine nucleotide and nucleoside

levels and (b) purine nucleotides and nucleosides are involved in and influence a number of biological processes, modulation of the activity of NTDPases may have significant effects on such biological processes. Therefore, there exists a need for effective inhibitors of NTDPases, to better modulate the activity of NTDPases, thus modulating the levels of purine nucleotides and nucleosides, which in turn results in the modulation of a variety of biological processes.

SUMMARY OF THE INVENTION

An aspect of the present invention is a C8-substituted purine nucleotide analog, wherein the analog is substituted at the C8 position with a substituent other than H.

A further aspect of the present invention is a composition comprising the above-mentioned analog in admixture with a suitable diluent or carrier.

Yet a further aspect of the present invention is a method for modulating the activity of an NTPDase enzyme comprising exposing the enzyme to the above-mentioned analog or composition.

In a preferred embodiment, the present invention provides a method for inhibiting the activity of an NTPDase enzyme comprising exposing the enzyme to the above-mentioned analog or composition.

Yet a further aspect of the present invention is a method for modulating the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in a biological system, comprising the step of introducing into said system the above-mentioned analog or composition.

Yet a further aspect of the present invention is a method for modulating the activity of a biological process in a biological system, wherein said process is affected by the level of purine nucleotide(s) and/or nucleoside(s) and/or

metabolite(s) or derivative(s) thereof in said system, comprising the step of introducing into said system the above-mentioned analog or composition.

Yet a further aspect of the present invention is a use of the above-mentioned analog or composition for modulating the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in a biological system.

Yet a further aspect of the present invention is a use of the above-mentioned analog or composition for modulating the activity of a biological process in a biological system, wherein said process is affected by the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in said system.

Yet a further aspect of the present invention is a commercial package containing the above-mentioned analog or composition together with instructions for modulating the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in a biological system.

Yet a further aspect of the present invention is a commercial package containing the above-mentioned analog or composition together with instructions for modulating the activity of a biological process in a biological system, wherein said process is affected by the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in said system.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Structures of different ATP analogs.

Figure 2: Scheme of synthesis of 8-thioether-ATP derivatives. A. 10 eq. NaSH/wet DMF/100°C/overnight/100% yield. B. 1. compound 10/MeOH/0.25 M NaOH/RT, freeze drying. 2. alkyl

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bromide/DMF/60°C/overnight/83-93% yield. C. 1. POCl₃/proton sponge. 2. P₂O₇H₂(Bu₃NH⁺)₂. 3. 0.2 M TEAB.

Figure 3: Hydrolysis of ATP and analogs by NTPDase.

5 ATP and analogs were used at a concentration of 100 μM. Hydrolysis was carried out at 37°C for 7 min in the presence of 1.9 μg of protein. C2 substituted analogs (2a-d) are all hydrolyzed by the enzyme whereas C8-substituted analogs (6-8) are more or less resistant to NTPDase hydrolysis. Results are
10 expressed as the mean ± SEM of n replicate (see figure) carried out in triplicate.

Figure 4: Kinetic parameters of bovine spleen NTPase for ATP, ADP and purine analogs. Experiments were carried out
15 in triplicate and results are expressed as the mean ± SEM of the best fit obtained with GraFit 4 software (Erithacus, UK). Apparent Km and Vmax were estimated from Eadie and Hofstee representation and Ki's from Dixon plots.

20 **Figure 5: Inhibitory effect of 8-BuS-ATP, 6e, on ATP** hydrolysis by NTPDase. A) Lineweaver-Burk representation of NTPDase inhibition. ATP (10 to 100 μM) and 8-BuS-ATP: 0 μM : ♦; 10 μM : ■; 25 μM : ▲; 50 μM : ●; and 100 μM : X. B) Dixon plots of NTPDase inhibition. ATP as substrate: 10 μM (▲), 25 μM (■) and 50 μM (♦) and 8-BuS-ATP concentrations as above. 8-BuS-ATP
25 produce a competitive inhibition with an estimated Ki of 10 μM. Results are expressed as the mean ± SEM of two experiments each in duplicate.

30 **Figure 6: Dixon representations of 8-CH₂tBuS-ATP, 6b,** (panel A) and 8-cycloheptylS-ATP, 6a, (panel B) inhibition. ATP concentration: 50 μM (♦) and 100 μM (■). Analog concentrations (0 to 100 μM). Both analogs act as mixed type inhibitors with estimated Ki of 45 and 31 μM for 8-tBuCH₂S-ATP,

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6b, and 8-cycloheptylS-ATP, 6a, respectively. Experiments were carried out in triplicate and expressed as the mean \pm SEM.

Figure 7: A) Lineweaver-Burk representation of the hydrolysis of 8-thioethyl-ATP, 6c, by NTPDase. B) Dixon representations of 8-thiohexyl-ATP, 6d, inhibition. ATP concentration: 15 μ M (\blacklozenge) and 50 μ M (\blacksquare). Analog concentrations (0 to 100 μ M). Analog 6d acts as a non-competitive inhibitor with an estimated K_i of 16 μ M. Experiments was carried out in triplicate and expressed as the mean \pm SEM.

Figure 8: Purinergic activity of 8-BuS-ATP, 6e.

A) Effect of 8-BuS-ATP (0.1-1000 pmol) on denuded mesenteric bed of guinea pig. Results are expressed as % of control (control = 37 mm Hg). No variations of perfusion pressure were measured. Results are the mean \pm SEM of at least three experiments.

B) Effect of 8-BuS-ATP on the relaxing effect of ATP on intact mesenteric bed. ATP (0.1-10000 pmol) in the presence of 7 μ M of 8-BuS-ATP (open bars) or in absence (control: closed bars). Results are expressed as % of vasodilation measured from a precontracted vessel (200 μ M of noradrenaline). No significant difference was observed. Results are the mean \pm SEM of at least three experiments.

C) Vascular responses with or without endothelium. Endothelium integrity was tested with 100 pmol of NK-1 (closed bar) and blood vessel responsiveness was evaluated by 3 nmol of NaNP (open bar). Results are expressed as % of vasodilation measured from a precontracted vessels (200 μ M of noradrenaline). In denuded mesentery there is significant response to NK-1. Results are the mean of three experiments or more.

DESCRIPTION OF THE EMBODIMENTS OF THE INVENTION

The present invention provides C8-substituted purine nucleotide analogs. In a preferred embodiment, such analogs
5 are ATP analogs, examples of which the Applicant has prepared and characterized. Such analogs have a variety of uses, a preferred one of which is the inhibition of NTPDases, which the Applicant has characterized. For this use, the compounds of the invention were found to be effective.

10 The Applicant has selected and synthesized purine nucleotide analogs, more particularly ATP analogs, specifically compounds 2a-d and 6-8 (illustrated in Figures 1 and 2). Based on the promising hydrolytic stability of compounds 6-8, the Applicant has synthesized a new series of 8-thioether ATP
15 analogs, i.e., compounds 6a-e, and has evaluated their use as NTPDase inhibitors. In view of finding a specific and potent NTPDase inhibitor, the Applicant has examined two series of ATP analogs substituted at positions C2 and C8, respectively.

As described in the Examples below, this evaluation
20 revealed that at least one of the compounds tested was improved with respect to, for example, at least one criterion selected from the following:

1. Resistance to NTDPase hydrolysis.
- 25 2. Potency as an NTDPase inhibitor.
3. Nature of the inhibition.
4. K_i value.
5. Effects on P2 (e.g. P2X and P2Y) purinergic
receptors.

30 Via the above evaluation, the Applicant has first demonstrated that analogs substituted with electron donating groups at C8 were more resistant to NTPDase hydrolysis than the corresponding C2 substituted analogs. Therefore, an aspect of

the invention are C8-substituted purine nucleotide analogs, a preferred embodiment of the invention being ATP analogs.

The C8-substituted purine nucleotide analogs of the invention may be substituted at this position, for example, by
5 electron donating groups. Such groups include but are not limited to ethers, thioethers and amines. Examples of ethers, thioethers and amines that are aspects of the invention are those with, for example, alkyl groups. Such alkyl groups may be, for example, cyclic-, branched- and/or n-alkyl groups. An
10 example of a preferred cyclic alkyl group according to the invention is the cycloheptyl (C_7H_{13}) group. An example of a preferred branched alkyl group according to the invention is the 2,2-dimethyl-propyl ($(CH_3)_3CCH_2$) group. Examples of preferred n-alkyl groups according to the invention are those
15 up to 6 carbons in length. A particularly preferred embodiment is an n-butyl ($CH_3(CH_2)_3$) group.

The Applicant has further demonstrated that of the C8-substituted ATP analogs analyzed, all of which were found to possess a degree of resistance to NTPDase hydrolysis, compounds
20 **6a**, **6b**, **6d** and **6e** were more resistant to hydrolysis by NTPDase than compounds **7**, **8** and **6c**. The Applicant has further evaluated the C8-substituted ATP analogs compounds **6a**, **6b**, **6d** and **6e** of the invention for their potency as NTPDase inhibitors, and has demonstrated that all four of these
25 compounds are good inhibitors. Therefore, compounds **6a**, **6b**, **6d** and **6e** are further preferred aspects of the present invention.

Of all the compounds tested, compound **6e** was found to be most resistant to hydrolysis by NTPDase, and was further found to exhibit competitive inhibition with a K_i value lower
30 than those measured for compounds **6a**, **6b** and **6d**. Therefore, as noted above, the Applicant notes that compounds **6a**, **6b**, **6d** and **6e** are further preferred aspects of the present invention, and compound **6e** represents a particularly preferred aspect of the invention.

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The Applicant has further evaluated compound 6e of the invention with respect to any effects on purinoceptors, and found that this compound does not interact with either P2X- or P2Y-purinoceptors. Therefore, the compounds of the invention
5 act as, for example, effective and specific inhibitors of NTPDase.

Therefore, the invention provides C8-substituted purine nucleotide analogs, which may, for example, be used for the modulation of NTPDase activity. In a preferred embodiment,
10 the C8-substituted purine nucleotide analogs of the invention may be used for the inhibition of NTPDase activity. Given the wide variety of biological processes which are affected by the purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives, the compounds of the invention may be
15 utilized to alter the activity of such processes via the alteration of the levels of purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives.

Accordingly, an aspect of the present invention is a method to alter or modulate the level of purine nucleotides
20 and/or nucleosides and/or their metabolites and/or derivatives in a biological system. A further aspect of the present invention is a method of altering the activity of a biological process which is affected by the levels of purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives
25 in a biological system. The modulation of such processes occurs by, for example, the action of purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives on cell surface receptors, such as purinoceptors. Such cell surface receptors can act to modulate a large number of biological
30 processes using a variety of mechanisms. Examples of such mechanisms include acting through G-proteins to generate a variety of signalling cascades (e.g., involving inositol phospholipid or other messengers and/or the mobilization of calcium stores), the activation of ligand-gated ion channels,

the induction of channels and/or pores, and the modulation of ion fluxes and other responses.

The processes noted above which may be modulated as a result of the modulation of the levels of purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives include, but are not limited to, the following:

1. In the cardiovascular system, such processes include, for example, platelet aggregation; modulation of vascular tone and function (e.g., vasoconstriction and vasodilation) and blood flow; heart function and performance; and the recruitment and adhesion of blood cells involved in inflammatory processes.

2. In the nervous system (central and peripheral), such processes include, for example, neurotransmission.

3. In sensory systems, such processes include, for example, activity of sensory organs and/or cells.

4. In muscle tissues, such processes include, for example, activity (e.g., contractile responses) of visceral smooth muscle and skeletal muscle.

5. In the pulmonary system, such processes include, for example, secretion by cells of the pulmonary system.

6. In the immune system, such processes include, for example, function of various immune cell types, and the modulation of diverse responses of the immune system.

7. In endocrine, neurocrine and exocrine systems, such processes include, for example, secretion of a variety of compounds from a variety of cell types in these systems. Such cell types include, for example, those present in the pancreas, and parotid, lacrimal, thyroid, adrenal and pituitary glands.

8. In paracrine cells, such as platelet and mast cells, such processes include, for example, secretion of compounds from these cells. Further, as noted above, the processes affected also include platelet aggregation, and

thrombus formation, as well as the additional recruitment of new platelets to the developing thrombus.

9. In the reproductive system and cells of the reproductive system, such processes include, for example, contraction of smooth muscle tissue (e.g., myometrium) and activity of germ cells.

10. In hepatic tissue, such processes include, for example, hepatic tissue function and the biochemical and biological processes which occur therein (e.g. gluconeogenesis and glycogenolysis), as well as the secretion of compounds (e.g., thromboxanes and prostaglandins) from hepatic cells and tissue.

11. In renal and gastrointestinal tissues, such processes include, for example, secretion (e.g., of electrolyte(s) and stomach acid) from such tissues and cells therein, and gastrointestinal motility.

12. In connective tissue, skin and bone, such processes include, for example, the modulation of a variety of activities and functions within these tissues and cells therein, such as growth and differentiation.

13. In tumor cells, such processes include, for example, a variety of activities and functions, such as growth regulation. Therefore, the growth of tumor or cancer cells and tissue may be modulated, and thus the invention further provides compounds, compositions, methods, uses, and commercial packages for the treatment of cancer.

Therefore, the invention provides compounds, compositions, methods, uses, and commercial packages for the modulation of activities and function in the cardiovascular, nervous, immune, inflammatory, sensory and reproductive systems; in muscle, endocrine, neuroendocrine, exocrine, paracrine, germ, hepatic, renal and gastrointestinal cells and tissues; as well as in connective tissue, skin and bone. The invention further provides compounds, compositions, methods,

uses, and commercial packages for the modulation of a process such as aggregation and thrombogenicity. In an embodiment, such modulation comprises an increase in aggregation and thrombogenicity.

5 Yet a further aspect of the present invention is the use of a compound of the invention for, for example, the methods and purposes described above.

The compounds of the present invention may also be adapted for certain applications using methods known in the
10 art. For example, a compound of the invention may be attached to a solid phase or matrix. In this form, the compound may be utilized for the isolation and purification of species with which it binds/interacts, for example, using the technique of affinity chromatography. Such species comprise proteins which
15 bind purine nucleotides and/or their metabolites and/or derivatives, an example of such a protein being an NTPDase enzyme. In other embodiments, the compounds of the invention may be modified for their use in a variety of methods such as diagnostic methods.

20 The compounds of the invention or corresponding modified versions may be a component of an appropriate composition of the invention, comprising a compound of the invention or a corresponding modified version and a suitable diluent or carrier. Such compositions may be, for example,
25 utilized in the uses and methods described above. Such compositions include pharmaceutical compositions, comprising a compound of the invention or a corresponding modified version and a suitable pharmaceutically acceptable diluent or carrier. The compounds, corresponding modified versions, or the
30 compositions of the invention may also be a component of a commercial package of the invention, which comprises a compound or composition of the invention together with instructions for, for example, the uses and methods described above.

The following examples are provided in order to illustrate the embodiments of the present invention and are not meant to limit the scope of the invention.

5 **Example 1: Synthesis, purification and characterization of ATP analogs.**

General methods. New compounds were characterized by proton and carbon nuclear magnetic resonance using a Bruker AC-200 or DPX-300 spectrometer. The chemical shifts are reported
10 in ppm relative to TMS as an internal standard. Nucleotides were characterized also by ^{31}P -NMR in D_2O using 85% H_3PO_4 as an external reference on a Bruker AC-200 spectrometer. Mass spectra were recorded on an AutoSpec-E-FISION VG high resolution Mass Spectrometer. Nucleotides were characterized
15 by FAB (fast atom bombardment) and high resolution FAB using a glycerol matrix under FAB negative conditions on AutoSpec-E-FISION VG high resolution Mass Spectrometer. Separation of the newly synthesized nucleotides was achieved using LC (Isco UA-6) using DEAE A-25 Sephadex (HCO_3^- form) anion exchanger as
20 described below. Final purification was done using an HPLC (Merck-Hitachi) system using a semi-preparative LiChroCART LiChrospher 60 RP-select B column (1 x 25 cm, Merck KGaA) and a linear gradient of 0.1 M triethylammonium acetate buffer (TEAA, pH 7.5) and methanol (see below) at 6 mL/min flow rate. For
25 analytical purposes, a LiChroCART LiChrospher 60 RP-select B column (250 mm x 4.6 mm, Merck KGaA) was used applying the same gradient as above at 1 mL/min flow rate. The purity of the nucleotides described below was evaluated on an analytical column in two different solvent systems. One solvent system
30 (I) was 0.1 TEAA/ CH_3OH , 80:20 to 20:80 in 20 min. The second solvent system (II) was (A) 5 mM tetrabutylammonium phosphate (TBAP) in methanol and (B) 60 mM ammonium phosphate and 5 mM

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TBAP in 90% water/10% methanol, applying a gradient of 25% A to 75% A in 20 min.

The selection and synthesis of ATP analogs, compounds 2a-d and 6-8 (Figures 1 and 2), for their evaluation as NTPDase inhibitors were based on the promising hydrolytic stability of compounds 6-8. The desired derivatives, 6a-e, were obtained in three steps from 8-Br-adenosine in good yields (Figure 2). 8-Mercapto-adenosine, 10, was obtained in a quantitative yield from 8-Br-adenosine upon treatment with 10 eq of NaSH in wet DMF at 100°C overnight. The corresponding dry sodium thiolate salt, obtained upon dissolution of 10 in MeOH/0.25 M NaOH and subsequent freeze drying, was treated with the appropriate alkyl bromide in DMF at 60°C to yield compounds 11 in high yields. Finally these compounds were 5'-triphosphorylated, to give nucleotides 6 in reasonable yields (64). Compound 6 of Figure 1E and compound 6e of Figure 2 are one in the same, i.e., with a thiobutyl ($\text{CH}_3(\text{CH}_2)_3\text{S}$) group as the C8 substituent. This compound was prepared as described previously (64).

8-Mercaptoadenosine (10). NaSH (0.8 g, 10 eq) was added to a solution of 8-bromoadenosine (0.5 g, 1.44 mmol) in DMF (7 mL). The mixture was warmed to 100°C and a few drops of water were added to improve solubility. The mixture was stirred at 100°C overnight. The solvent was evaporated under high vacuum and the residue was coevaporated repeatedly with MeOH, until the residue turned into a solid. The residue was dissolved in water and neutralized with NaOH. After freeze drying, the product was purified on a silica gel column (CHCl_3 :MeOH 10:1). The product was obtained as a yellowish powder (100% yield, mp 169-170 °C). $^1\text{H-NMR}$ (CD_3OD , 200 MHz) 8.09 (s, 1H, H-2), 6.65 (d, $J = 7$ Hz, 1H, H-1'), 5.01 (dd, $J = 7, 5.5$ Hz, 1H, H-2'), 4.39 (dd, $J = 5.5, 2.5$ Hz, 1H, H-3'), 4.13 (q, $J = 2.5$ Hz, 1H, H-4'), 3.87 (dd, $J = 12.5, 2.5$ Hz, 1H, H-5'), 3.71

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(dd, $J = 12.5$, 3 Hz, 1H, H-5'); ^{13}C -NMR (CD_3OD , 300 MHz) δ 167.88 (C-6), 151.92 (C-2), 148.12 (C-4), 147.88 (C-8), 107.00 (C-5), 88.62 (C-1'), 85.59 (C-4'), 70.70 (C-2'), 70.62 (C-3'), 62.13 (C-5'); MS (CI/ NH_3): m/z 317 $\text{M}+\text{NH}_4^+$.

5

8-(Thiocycloheptyl) adenosine (11a). A suspension of 8-mercaptoadenosine (75 mg, 0.25 mmol) in MeOH (2 mL) was dissolved in 0.25 M NaOH (1 mL). The clear, yellow solution was stirred at room temperature for 1 h. After freeze drying, the thiolate sodium salt obtained as a yellowish solid, was dissolved in dry DMF (2 mL) and bromocycloheptane (38 μL , 1.1 eq) was added. The solution was stirred overnight under nitrogen at 60°C. The solvent was evaporated under high vacuum and the yellow residue was coevaporated repeatedly with MeOH, until the residue turned into a yellow solid. The solid was triturated with petroleum ether/ether 1:1, and then separated on a silica gel column (CHCl_3 :MeOH 20:1). Product **11a** was obtained as a white solid in 83 % yield (82 mg) after evaporation and drying, mp 205-6 °C. ^1H -NMR ($\text{DMSO}-d_6$, 200 MHz): 8.07 (s, 1H, H-2), 7.36 (br.s, 2H, NH_2), 5.84 (d, $J = 7\text{Hz}$, 1H, H-1'), 5.00 (dd, $J = 7$, 5 Hz, 1H, H-2'), 4.16 (dd, $J = 5$, 2 Hz, 1H, H-3'), 4.07-3.91 (m, 2H, H-4' & SCH), 3.68 (dd, $J = 12$, 4 Hz, 1H, H-5'), 3.51 (dd, $J = 12.5$, 4 Hz, 1H, H-5'), 2.19-1.93 (m, 2H), 1.84-1.42 (m, 11H); ^{13}C -NMR ($\text{DMSO}-d_6$, 300 MHz): δ 154.80 (C-6), 151.51 (C-2), 150.03 (C-4), 147.94 (C-8), 119.75 (C-5), 88.96 (C-1'), 86.64 (C-4'), 71.31 (C-2'), 71.08 (C-3'), 62.27 (C-5'), 48.42 (SCH), 34.51 (CH_2), 34.14 (CH_2), 27.77 (CH_2), 27.76 (CH_2), 25.14 (CH_2), 25.04 (CH_2). FAB(positive): m/z 396 MH^+ .

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8-(Thio-2,2-dimethyl-propyl)-adenosine (11b). The compound was prepared as described for 8-(thiocycloheptyl)-adenosine and obtained in 79 % yield (73 mg) as a yellowish

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solid, mp 141-2°C. ¹H-NMR (CD₃OD, 200 MHz) δ 8.06 (s, 1H, H-2), 6.02 (d, J = 7 Hz, 1H, H-1'), 4.99 (dd, J = 7, 5 Hz, 1H, H-2'), 4.33 (dd, J = 5, 2 Hz, 1H, H-3'), 4.17 ("q", J = 2 Hz, 1H, H-4'), 3.88 (dd, J = 12.5, 2.5 Hz, 1H, H-5'), 3.72 (dd, J = 12.5, 3 Hz, 1H, H-5'), 3.42 and 3.35 (AB, J = 7 Hz, 2H, SCH), 1.09 (s, 9H, SCH₂(CH₃)₃); ¹³C-NMR (CD₃OD, 300 MHz) 155.92 (C-6), 152.91 (C-4), 152.11 (C-2), 151.69 (C-8), 110.64 (C-5), 91.19 (C-1'), 88.89 (C-4'), 74.13 (C-2'), 73.20 (C-3'), 64.17 (C-5'), 47.69 (SCH₂), 29.07 (3C, SCH₂(CH₃)₃); MS (CI/NH₃) m/z: 368 (M-H)⁺.

10

8-(Thioethyl)-adenosine (11c). A suspension of 8-mercaptoadenosine (270 mg, 0.9 mmol, in 7 mL MeOH) was dissolved in 0.25 M NaOH (3.6 mL). The clear, yellow solution was stirred at room temperature for 1 h. After freeze drying, the thiolate sodium salt, obtained as a yellowish solid, was dissolved in dry DMF (3 mL) and bromoethane (101 μL, 1.5 eq) was added. The solution was stirred under nitrogen at room temperature for 3 h. The solvent was evaporated under high vacuum and the yellow residue was coevaporated repeatedly with MeOH, until the residue turned into a yellow solid. The solid was separated on a silica gel column (CHCl₃:MeOH 15:1). Product 11c was obtained as a white solid in 93 % yield after evaporation and drying (273 mg), mp 176°C. ¹H-NMR (DMSO-d₆, 200 MHz): 8.05 (s, 1H, H-2), 7.31 (br. s, 2H, NH₂), 5.76 (d, J = 7 Hz, 1H, H-1'), 5.66 (dd, J = 8.5, 3.5 Hz, 1H, OH-5'), 5.42 (d, J = 6 Hz, 1H, OH-2'), 5.21 (d, J = 4 Hz, 1H, OH-3'), 4.99 (br. q, J = 6 Hz, 1H, H-2'), 4.15 (bs, 1H, H-3'), 3.96 (br. s, 1H, H-4'), 3.68 (dt, J = 12, 3.5 Hz, 1H, H-5'), 3.60-3.44 (m, 1H, H-5'), 3.43-3.14 (m, 1H, SCH₂CH₃), 1.36 (t, J = 7 Hz, 3H, SCH₂CH₃); ¹³C-NMR (DMSO-d₆, 300 MHz): 154.49 (C-6), 151.30 (C-2), 150.40 (C-4), 148.53 (C-8), 119.66 (C-5), 88.86 (C-1'), 86.63 (C-4'), 71.29 (C-2'), 70.01 (C-3'), 62.24 (C-5'), 26.77 (SCH₂CH₃), 14.85 (SCH₂CH₃); MS (CI/CH₄)

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: m/z 328 MH⁺; High resolution MS: calcd for C₁₂H₁₈N₅O₄S
328.1079, Found 328.1069.

8-(Thio-n-hexyl)-adenosine (11d). The compound was
5 prepared as described for 8-(thioethyl)-adenosine and obtained
in 91 % yield (314 mg) as a white solid, mp 169-171°C. ¹H-NMR
(DMSO-d₆, 200 MHz): δ 8.05 (s, 1H, H-2), 7.29 (br.s, 2H, NH₂),
5.77 (d, J = 7 Hz, 1H, H-1'), 5.67 (dd, J = 9, 3.5 Hz, 1H, OH-
5'), 5.42 (d, J = 6 Hz, 1H, OH-2'), 5.21 (d, J = 4 Hz, 1H, OH-
10 3'), 4.99 ("q", J = 6 Hz, 1H, H-2'), 4.15 (br.s, 1H, H-3'), 3.96
(br.s, 1H, H-4'), 3.67 (dt, J = 12, 3.5 Hz, 1H, H-5'), 3.60-3.43
(m, 1H, H-5'), 3.42-3.18 (m, 1H, SCH₂), 1.69 (quint, J = 7 Hz,
2H, SCH₂CH₂), 1.50-1.18 (m, 6H, -CH₂CH₂CH₂CH₃), 0.86 (t, J = 7
Hz, 3H, CH₃); ¹³C-NMR (DMSO-d₆, 300 MHz): 154.55 (C-6), 151.27
15 (C-2), 150.38 (C-4), 148.71 (C-8), 119.61 (C-5), 88.85 (C-1'),
86.63 (C-4'), 71.28 (C-2'), 71.02 (C-3'), 62.23 (C-5'), 32.37
(SCH₂), 30.70 (CH₂), 28.80 (CH₂), 27.71 (CH₂), 21.99 (CH₂), 13.88
(CH₃); MS (CI/CH₄) : m/z 384 MH⁺; High resolution MS: calcd for
C₁₆H₂₆N₅O₄S 384.1705, Found 384.1696.

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Nucleoside 5'-Triphosphorylation. Nucleosides 11a-d
were 5'-triphosphorylated according to a published procedure
(60).

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8-(Thiocycloheptyl)-adenosine 5'-triphosphate (6a).

The compound was obtained in 60 % yield (79 mg). Final
separation was achieved on HPLC applying a linear gradient of
TEAA/ CH₃OH 70:30 to 20:80 in 20 min (6 mL/min). t_R 9.53 min.

¹H-NMR (D₂O, 200 MHz): δ 8.17 (s, 1H, H-2), 6.09 (d, J = 6 Hz,
30 1H, H-1'), 5.15 (t, J = 6 Hz, 1H, H-2'), 4.62-4.51 (m, 1H, H-
3'), 4.37-4.14 (m, 3H, H-4' & H-5'), 3.93-3.75 (m, 1H, SCH),
2.15-1.90 (m, 2H), 1.80-1.36 (m, 11H); ³¹P-NMR (D₂O, 200 MHz, pH

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9) δ -5.34 (d), -10.37 (d), -21.32 (t); UV: λ_{\max} 282 nm. HRFAB: calcd for $C_{17}H_{27}N_5O_{13}P_3S$ 634.0539, Found 634.0540; t_R 14.99 min (95 % purity) using solvent system I, 13.19 min (97 % purity) using solvent system II.

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8-(Thio-2,2-dimethyl-propyl)-adenosine 5'-triphosphate

(6b). The compound was obtained in 65 % yield (77 mg). Final separation was achieved on HPLC applying a linear gradient of TEAA/ CH_3OH 70:30 to 20:80 in 20 min (6 mL/min). t_R 7.51 min.

10 1H -NMR (D_2O , 200 MHz): δ 8.21 (s, 1H, H-2), 6.13 (d, J = 6 Hz, 1H, H-1'), 5.20 (t, J = 6 Hz, 1H, H-2'), 4.62 (dd, J = 6 Hz, 1H, H-3'), 4.42-4.25 (m, 3H, H-4' & H-5'), 3.29 and 3.35 (ABq, J = 12 Hz, 2H, SCH_2), 1.06 (s, 3H, $SCH_2(CH_3)_3$); ^{31}P -NMR (D_2O , 200 MHz, pH 9) δ -10.23 (d), -10.79 (d), -22.61 (t); UV: λ_{\max} 282
15 nm. HRFAB: calcd for $C_{15}H_{25}N_5O_{13}P_3S$ 608.0382, Found 608.0360; t_R 12.71 min (96 % purity) using solvent system I, 11.31 min (95 % purity) using solvent system II.

8-(Thioethyl)-adenosine 5'-triphosphate (6c). The

20 compound was obtained in 43 % yield (84 mg). Final separation was achieved on HPLC applying a linear gradient of TEAA/ CH_3OH 90:10 to 20:80 in 20 min (6 mL/min). t_R 8.11 min. 1H -NMR (D_2O , 200 MHz): 8.15 (s, 1H, H-2), 6.10 (d, J = 6.5 Hz, 1H, H-1'), 5.17 (t, J = 6.5 Hz, 1H, H-2'), 4.65-4.55 (m, 1H, H-3'), 4.40-
25 4.16 (m, 3H, H-4' & H-5'), 3.30 and 3.26 (ABq of t, J = 11.5, 7 Hz, 1H each, SCH_2), 1.39 (t, J = 7 Hz, 3H, CH_3); ^{31}P -NMR (D_2O , 200 MHz, pH 9) -5.12 (d), -10.31 (d), -20.98 (t); UV: max 282 nm. HRFAB: calcd for $C_{12}H_{18}N_5O_{13}NaP_3S$ 587.9732, Found 587.9650; t_R 7.16 min (96 % purity) using solvent system I, 3.55 min (94 %
30 purity) using solvent system II.

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8-(Thio-n-hexyl)-adenosine 5'-triphosphate (6d). The compound was obtained in 58 % yield (111 mg). Final separation was achieved on HPLC applying a linear gradient of TEAA/ CH₃OH 70:30 to 20:80 in 20 min (6 mL/min). t_R 10.73 min. ¹H-NMR (D₂O, 200 MHz): 8.17 (s, 1H, H-2), 6.01 (d, J = 6.5 Hz, 1H, H-1'), 5.19 (t, J = 6.5 Hz, 1H, H-2'), 4.64-4.55 (m, 1H, H-3'), 4.40-4.14 (m, 3H, H-4' & H-5'), 3.32 and 3.24 (ABq of t, J = 14, 7 Hz, 1H each, SCH₂), 1.73 ("quint", J = 7 Hz, 2H, SCH₂CH₂), 1.52-1.09 (m, 6H), 0.82 (t, J = 7 Hz, 3H, CH₃); ³¹P-NMR (D₂O, 200 MHz, pH 9) -5.12 (d), -10.25 (d), -21.03 (t); UV: max 282 nm; t_R 15.25 min (>97 % purity) using solvent system I, 15.52 min (>97 % purity) using solvent system II.

Example 2: Enzymology of purine nucleotide analogs

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The demonstration of the inhibitory properties of a given compound on a particular enzyme requires that a single site of catalysis is present in the medium. In other words, if a second enzyme competes for the substrate or generates the same reaction product it modifies the interpretation and would definitely rule out any conclusion about the inhibitor specificity. In the preparation used to demonstrate the inhibitory properties of C8-substituted ATP analogs, in this work, we previously showed that a single catalytic site was involved in the sequential hydrolysis of the gamma and beta phosphate residues of ATP (35).

Reagents and solutions. ATP, tetramisole, malachite green, bovine serum albumin fraction V (BSA), CHAPS, sodium nitroprussiate (NaNP), (-) arterenol bitartrate (noradrenaline), heparin and indomethacin were obtained from Sigma Chemical Co. (St-Louis, MO, USA). ADP was obtained from Roche (Laval, QC., Canada), and Bradford reagent was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). [Sar⁹, Met (O₂)¹¹]SP

(NK-1) was synthesized by Dr. W. Neugebauer from the Université de Sherbrooke. Preparation of Krebs and phosphate buffer saline (PBS) were as followed. Krebs solution: 5.5 mM glucose, 117.5 mM NaCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 4.7 Mm KCl, 2.5 Mm CaCl₂, 25 Mm NaHCO₃, pH 7.4. PBS: 137 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄ and 1.7 mM KH₂PO₄, pH 7.4. All the other reagents were of analytical grade and obtained from Sigma Chemical Co. (St-Louis, MO, USA).

10 **Isolation of particulate fractions.** Experiments were carried out with a particulate fraction obtained from bovine spleen according to the method of Sévigny et al. (37). Briefly, bovine spleens were cut in small pieces and homogenized (15-20%) with a Polytron in Tris-saline buffer supplemented with SBTI and
15 PMSF as protease inhibitors. After filtration with cheesecloth and centrifugation at 600 g the supernatant is centrifuged at 22,000 g for 90 min, and the resulting pellet is suspended in bicarbonate buffer and loaded on a sucrose cushion (40%) and centrifuged for two hours at 100,000 g. The fraction floating
20 on the cushion is harvested in five volumes of bicarbonate buffer. The pellet is suspended in Tris buffer/glycerol 7,5% and kept at -20°C.

NTPDase assays. Enzyme activity was routinely
25 measured by the release of inorganic phosphorus with the malachite green colorimetric assay (61). Resistance to hydrolysis was measured at 37°C in 1 ml of the following incubation medium: 8mM CaCl₂, 5 mM tetramisole, 50 mM Tris base, 50 mM imidazole, buffered at pH 7.6, and 100 µM of either ATP
30 or its analogs. Apparent K_m and V_{max} values for ATP, ADP and each of the hydrolysable purine nucleotide analogs were derived from Eadie and Hofstee plots, with substrate concentrations ranging between 10 and 300 µM for ATP and ADP, and between 15 and 100 µM for the analogs, unless stated otherwise. In both

cases the reaction was started by the addition of 1.9 μ g of the enzyme preparation and stopped after 7 min with 250 μ L of the malachite green reagent. Apparent K_i values for non-hydrolysable purine nucleotide analogs were derived from Dixon replots, using inhibitor concentrations ranging from 0 to 100 μ M. Reactions were performed in the same incubation buffer, as previously described and were started by the addition of non-saturating ATP concentrations. Protein concentration was determined with the Bradford microplate assay using bovine serum albumin as a standard of reference (62).

To reduce potential artifacts resulting from the solubilisation of NTPDase by detergents, a particulate fraction (prepared as described above) was used. It is important to mention that there was no other detectable level of ATPase or ADPase activity in the preparation, other than that attributable to NTPDase. Also, possible alkaline phosphatase activity was inhibited by tetramisole added to the assays. In view of finding a specific and potent NTPDase inhibitor, two series of ATP analogs were examined, which were substituted at positions C2 and C8, respectively.

Analysis of NTPDase hydrolysis of ATP analogs. The ATP analogs substituted at positions C2 and C8, respectively, were first tested with respect to their resistance to NTPDase hydrolysis. The results obtained demonstrate that analogs substituted with electron donating groups at C8 were more resistant to hydrolysis than the corresponding C2 substituted analogs (Fig. 3). While a level of hydrolysis was observed with 8-Br-ATP, 8-BuNH-ATP, 7, 8-BuO-ATP, 8, and 8-ethylS-ATP, 6c (Fig. 7A), the compounds 8-cycloheptylS-ATP, 6a, 8-CH₂tBuS-ATP, 6b, 8-hexylS-ATP, 6d, and 8-BuS-ATP, 6e, were resistant to hydrolysis by NTPDase (Fig. 3). It is noteworthy that all the C2 substituted molecules displayed K_m values in the range found for ATP and ADP (Fig. 4). This indicates that the affinity for

the catalytic site is equivalent for all these analogs. Hence the position of the substituent is clearly important for resistance to the catalytic activity of the enzyme.

Hydrophobic interactions and H-bonds of the C2 substituent appear to be important determinants for P2Y-R ligand affinity. The conformational preference of the ligands in solution, determined by NMR experiments, may explain in part the differences in P2Y-R potency between the 2- and 8-substituted compounds. All 2-substituted derivatives possess an anti conformation, whereas the 8-ether and thioether analogs are in the syn conformation. The latter are apparently not tolerated by the tentative P2Y₁-R binding-site (64-66). In contrast, the NTPDase active site can accommodate 8-thioetherATP analogs, and even derivatives bearing large or bulky substituents (8-CH₂tBuS-ATP, and 8-cycloheptylS-ATP and 8-hexylS-ATP), probably in their expected syn conformation. This conformation is likely unfavorable for catalytic activity, namely, the orientation of the triphosphate chain in the syn conformation is probably shifted away from the catalytic amino acid residues.

Analysis of potency of C8-substituted ATP analogs as NTPDase inhibitors. Having established that these 8-thioether ATP derivatives were not significantly hydrolyzed, they were then investigated in regard to their potency as NTPDase inhibitors (Fig. 3). One striking feature is that the four derivatives, 6a, b, d, e are good inhibitors (Fig. 4-7). Again, the affinity (K_i) falls in the same range of affinity of that of ATP and ADP with one exception, the 8-CH₂tBuS-ATP, which has a slightly higher K_i (Fig. 4). However, even if these four analogs all display efficient NTPDase inhibition, 8-BuS-ATP, 6e, (Fig. 5) is to be considered as the preferred inhibitor, because it exhibits competitive inhibition with a K_i lower than those measured for compounds 6a, b, d, which are further less

stable analogs with respect to NTPDase hydrolysis. The fact that this inhibitor (6e) interacts specifically with the binding site of the enzyme potentially reduces the risk of interference with other ATP-binding enzymes or receptors, and thus possesses a high degree of specificity. 8-CH₂tBuS-ATP, 6b, and 8-cycloheptylS-ATP, 6a, showed mixed types of inhibition thereby complicating the interpretation of their interaction with NTPDase (Fig. 6). Analog 6d exhibits non-competitive inhibition, thereby suggesting that its inhibitory effects are the results of an interaction with another part of the enzyme distinct from the catalytic site (Fig. 7B). From these results, it appears that the catalytic-site of NTPDase, may tolerate long and bulky substituents at the C8 position and also tolerate nucleotides in syn and anti conformations. It is noteworthy that the electronic nature of the modified purine ring has almost no influence on the affinity for the catalytic site, since thioether, aminoether and oxyether shows similar apparent affinity (K_m or K_i) for the enzyme.

Example 3: Analysis of effects on P2-receptor activity of purine nucleotide analogs.

Surgical Procedures. Dunkin-Hartley guinea pigs (300-350 g) of either sex were sacrificed by cervical dislocation according to the Canadian Council on Animal Care. The guinea pig mesentery was prepared as described by Berthiaume et al (63). Briefly, the colic and ileocolic branches of the superior mesenteric artery were tied and the superior mesenteric artery cannulated (Portex size tube 3FG). To isolate the mesenteric bed from the intestine, the mesentery was perfused (2 ml/min, for 5 min) via the mesenteric artery with a Krebs solution containing heparin (100 U/ml). The mesentery was then separated by cutting close to the intestine. A resting period of 60 min was then allowed during which the guinea pig mesenteric bed was

perfused (2 ml/min) with a warmed (37°C) and gassed Krebs solution (95% O₂ and 5% CO₂) containing indomethacin (5 µM), as described earlier. In all the assays, perfusion pressure was increased to obtain a flow rate of 6 ml min⁻¹. Response of mesenteric bed, precontracted with noradrenaline (200 µM) in 0.9% saline solution, to the different drugs, was measured with a pressure transducer (Statham, model P-23AC) and recorded on a Grass physiograph (model 79D).

P2X-receptor assays. Guinea pig mesenteric bed was denuded from its endothelium layer by using 20 mM of CHAPS in PBS (63). Briefly, the CHAPS solution was infused for 45 sec, followed by a resting period of 30 min. Finally a second 45 sec infusion of 20 mM CHAPS was carried out. Blood vessels were then precontracted as earlier described. The efficiency of the endothelium removal technique was assessed by an intra-arterial bolus injection of 100 pmol of NK-1 in PBS. Reactivity of the media layer was confirmed by bolus injection of 3 nmol of NaNP (sodium nitroprussiate) in PBS. Bolus injections of increasing concentrations of 8-BuS-ATP (0.1 to 1000 pmol) in PBS, were administered. Variation of perfusion pressure were measured. Between each injection of 8-BuS-ATP a resting period was allowed to allow the return of pressure to baseline (i.e. precontracted pressure).

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P2Y-receptor assays. Intact mesenteric bed vessels were precontracted with noradrenaline (200 µM). Mesentery was infused for 7 min with 7µM of 8-BuS-ATP or PBS (control), followed by intra-arterial bolus injection of increasing concentrations of ATP (0.1 pmol to 10000 pmol). A resting period between each ATP injection was allowed to return to baseline, as described above. Blood vessel reactivity was assessed by a bolus injection of 3 nmol of NaNP. Response of

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the endothelium layer was confirmed by injection of NK-1 (100 pmol).

Statistics. Data are expressed as mean \pm SEM and number of replicates are in figure legends. Kinetic studies have been performed using Grafit software version 4 (Erithacus, UK). Unless stated otherwise, comparison between data was performed by one-way ANOVA test. Probability values of less than 0.05 were considered significant.

10

Effects on purinoceptor activity. Using the methods described above, the compounds of the invention were analyzed with respect to any effects on the activity of purinoceptors. Recent studies have indicated that 8-BuS-ATP was a poor agonist for P2Y₁ receptor. In rat astrocytes this compound has no effect on $[Ca^{2+}]_i$ level, whereas the corresponding 2-substituted ATP analogs potently increased $[Ca^{2+}]_i$ level. Similar results were obtained with turkey erythrocyte membranes (67). Since this molecule qualifies as a potent inhibitor, we tested its influence in the isolated mesenteric bed of the guinea pig for potential P2X and P2Y purinergic effects was tested (Fig. 8). Isolated guinea pig mesenteric artery and vein have been shown to respond to ATP via a P2X-purinoceptor located at the surface of the smooth muscle cells (68, 69). This latter purinoceptor was sensitive to α , β -CH₂-ATP, **1b**, a P2X agonist (70, 71). CHAPS was used to remove the blood vessel endothelium which gives rise to endothelium denuded vessels. Non-functional endothelium has been shown by a lack of response to 100 pmol of NK-1 (63). Our data show that 8-BuS-ATP was not able to initiate any P2X-R effect at concentrations up to 1000 pmol, indicating that it does not interact with P2X-R (Fig. 8A). We also evaluated the effect of an infusion of 8-BuS-ATP on the vasodilatory response induced by administration of increasing ATP concentrations (0.1 pmol to 10000 pmol), in intact

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mesenteric bed. Even in the presence of 7 μ M 8-BuS-ATP the response to ATP was unmodified, confirming that 8-BuS-ATP did not interact with P2Y-R (Fig. 8B).

The integrity of the endothelium was assessed and
5 confirmed with NK-1 and NaNP (Fig. 8C).

All of the references cited above and listed in the REFERENCES section below are herein incorporated by reference.

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CLAIMS:

1. A C8-substituted purine nucleotide analog, wherein the analog is substituted at the C8 position with a substituent
5 other than H.
2. The analog of claim 1, wherein the purine is adenine.
3. The analog of claim 1 or 2, wherein the substituent
10 is an ether, thioether or an amine.
4. The analog of claim 3, wherein the substituent is an ether, and wherein the ether substituent has the structure:
-O-X.
15
5. The analog of claim 4, wherein X is an alkyl group.
6. The analog of claim 5, wherein X is selected from the group consisting of:
20
 - (a) C_7H_{13} (cycloheptyl),
 - (b) $(CH_3)_3CCH_2$, and
 - (c) $CH_3(CH_2)_n$, wherein $1 \leq n \leq 5$.
7. The analog of claim 6, wherein the analog is
25 compound 8a.
8. The analog of claim 6, wherein the analog is compound 8b.
9. The analog of claim 6, wherein the analog is
30 compound 8c.
10. The analog of claim 6, wherein the analog is compound 8d.

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11. The analog of claim 6, wherein the analog is compound 8e.

12. The analog of claim 3, wherein the substituent is a thioether, and wherein the thioether substituent has the structure:

-S-X.

13. The analog of claim 12, wherein X is an alkyl group.

10

14. The analog of claim 13, wherein X is selected from the group consisting of:

(a) C_7H_{13} (cycloheptyl),

(b) $(CH_3)_3CCH_2$, and

15

(c) $CH_3(CH_2)_n$, wherein $1 \leq n \leq 5$.

15. The analog of claim 14, wherein the analog is compound 6a.

20 16. The analog of claim 14, wherein the analog is compound 6b.

17. The analog of claim 14, wherein the analog is compound 6c.

25

18. The analog of claim 14, wherein the analog is compound 6d.

19. The analog of claim 14, wherein the analog is compound 6e.

30

20. The analog of claim 3, wherein the substituent is an amine, and wherein the amine substituent has the structure:

-NH-X.

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21. The analog of claim 20, wherein X is an alkyl group.

22. The analog of claim 21, wherein X is selected from the group consisting of:

- 5 (a) C_7H_{13} (cycloheptyl),
(b) $(CH_3)_3CCH_2$, and
(c) $CH_3(CH_2)_n$, wherein $1 \leq n \leq 5$.

10 23. The analog of claim 22, wherein the analog is compound 7a.

24. The analog of claim 22, wherein the analog is compound 7b.

15 25. The analog of claim 22, wherein the analog is compound 7c.

26. The analog of claim 22, wherein the analog is compound 7d.

20

27. The analog of claim 22, wherein the analog is compound 7e.

28. A composition comprising the compound according to
25 any one of claims 1 to 27 in admixture with a suitable diluent or carrier.

29. A method for modulating the activity of an NTPDase
enzyme comprising exposing the enzyme to the analog according
30 to any one of claims 1 to 27 or the composition of claim 28.

30. A method for inhibiting the activity of an NTPDase
enzyme comprising exposing the enzyme to the analog according
to any one of claims 1 to 27 or the composition of claim 28.

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31. A method for modulating the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in a biological system, comprising the step of introducing into said system the compound according to
5 any one of claims 1 to 27 or the composition of claim 28.

32. A method for modulating the activity of a biological process in a biological system, wherein said process is affected by the level of purine nucleotide(s) and/or
10 nucleoside(s) and/or metabolite(s) or derivative(s) thereof in said system, comprising the step of introducing into said system the compound according to any one of claims 1 to 27 or the composition of claim 28.

15 33. The method of claim 32, wherein the biological process is aggregation and thrombogenicity.

34. Use of the analog according to any one of claims 1 to 27 or the composition of claim 28 for modulating the activity
20 of an NTPDase enzyme.

35. Use of the analog according to any one of claims 1 to 27 or the composition of claim 28 for inhibiting the activity
25 of an NTPDase enzyme.

36. Use of the compound according to any one of claims 1 to 27 or the composition of claim 28 for modulating the level of purine nucleotide(s) and/or nucleoside(s) and/or
metabolite(s) or derivative(s) thereof in a biological system.

30 37. Use of the compound according to any one of claims 1 to 27 or the composition of claim 28 for modulating the activity of a biological process in a biological system, wherein said process is affected by the level of purine

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nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in said system.

38. The use of claim 37, wherein the biological process
5 is aggregation and thrombogenicity.

39. A commercial package containing the compound
according to any one of claims 1 to 27 or the composition of
claim 28 together with instructions for modulating the activity
10 of an NTPDase enzyme.

40. A commercial package containing the compound
according to any one of claims 1 to 27 or the composition of
claim 28 together with instructions for inhibiting the activity
15 of an NTPDase enzyme.

41. A commercial package containing the compound
according to any one of claims 1 to 27 or the composition of
claim 28 together with instructions for modulating the level of
20 purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s)
or derivative(s) thereof in a biological system.

42. A commercial package containing the compound
according to any one of claims 1 to 27 or the composition of
25 claim 28 together with instructions for modulating the activity
of a biological process in a biological system, wherein said
process is affected by the level of purine nucleotide(s) and/or
nucleoside(s) and/or metabolite(s) or derivative(s) thereof in
said system.

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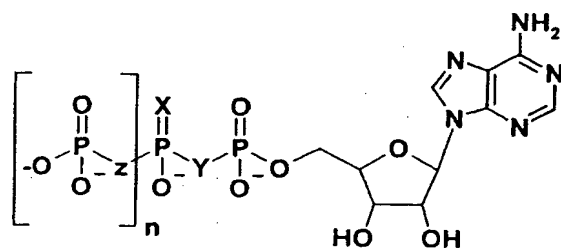
43. The commercial package of claim 42, wherein the biological process is aggregation and thrombogenicity.

5

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PATENT AGENTS

OTTAWA, CANADA

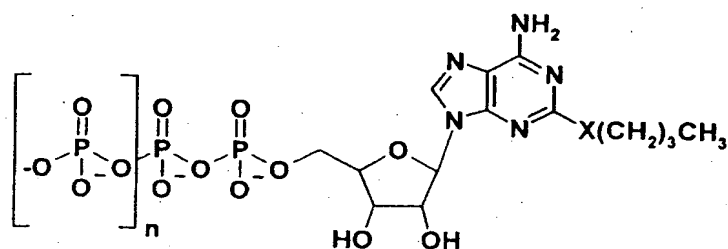
1/8



1 a : X = S, Y = O, Z = O, n = 0

b : X = O, Y = CH₂, Z = O, n = 1c : X = O, Y = O, Z = CH₂, n = 1d : X = S, Y = CH₂, Z = NH, n = 1

Fig. 1A



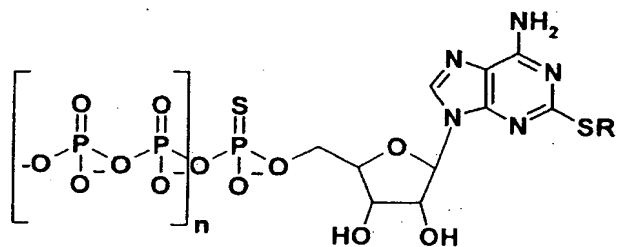
2 a : n = 1, X = S

b : n = 0, X = S

c : n = 1, X = NH

d : n = 1, X = O

Fig. 1B



3. n = 1, a : R = hexyl, b : R = benzyl

4. n = 0, a : R = hexyl, b : R = benzyl

Fig. 1C

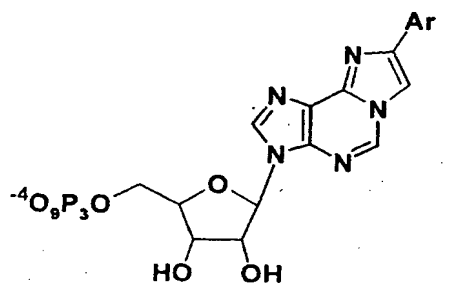
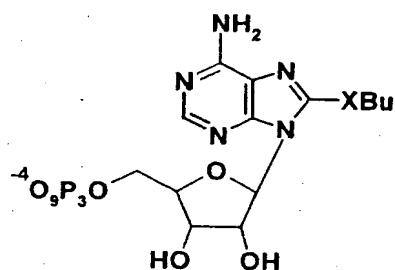
5 a : Ar = p-NO₂-C₆H₄b : Ar = p-NH₂-C₆H₄

Fig. 1D



6. X = S

7. X = NH

8. X = O

Fig. 1E

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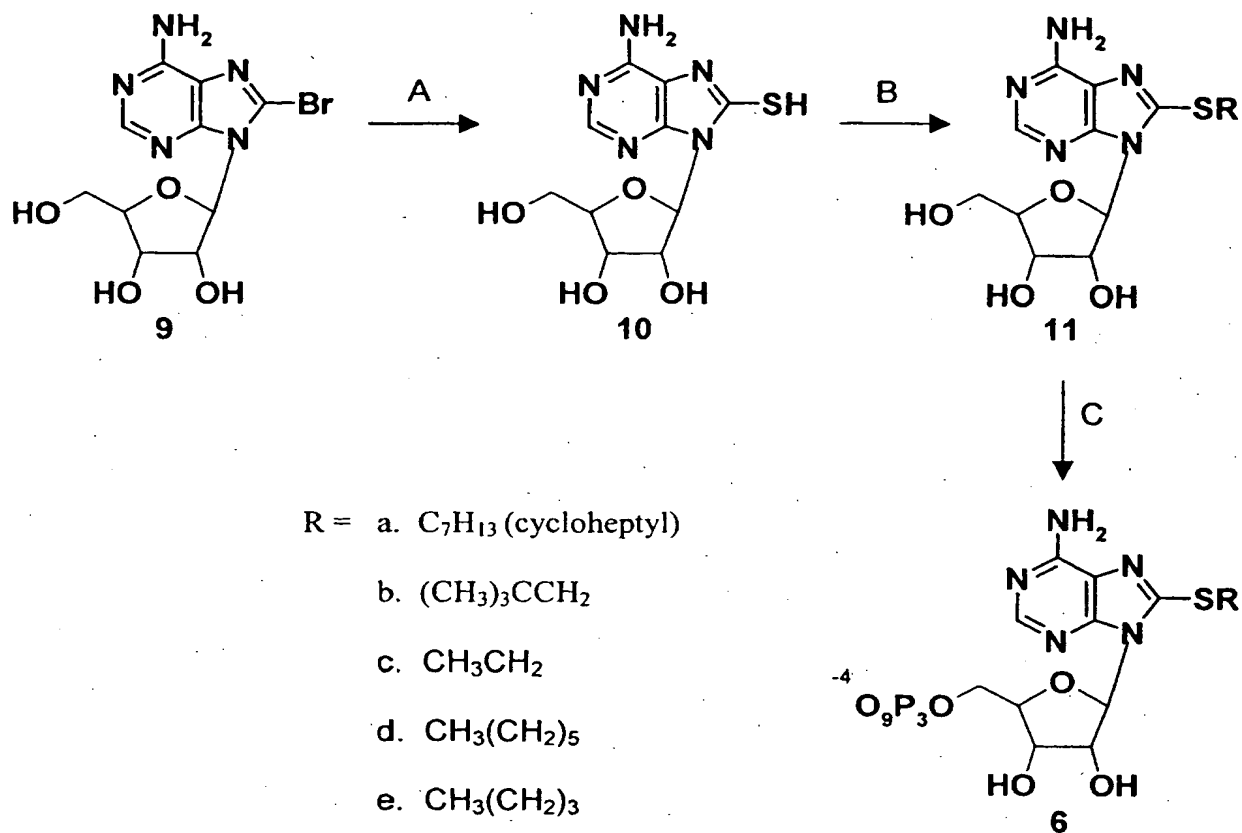
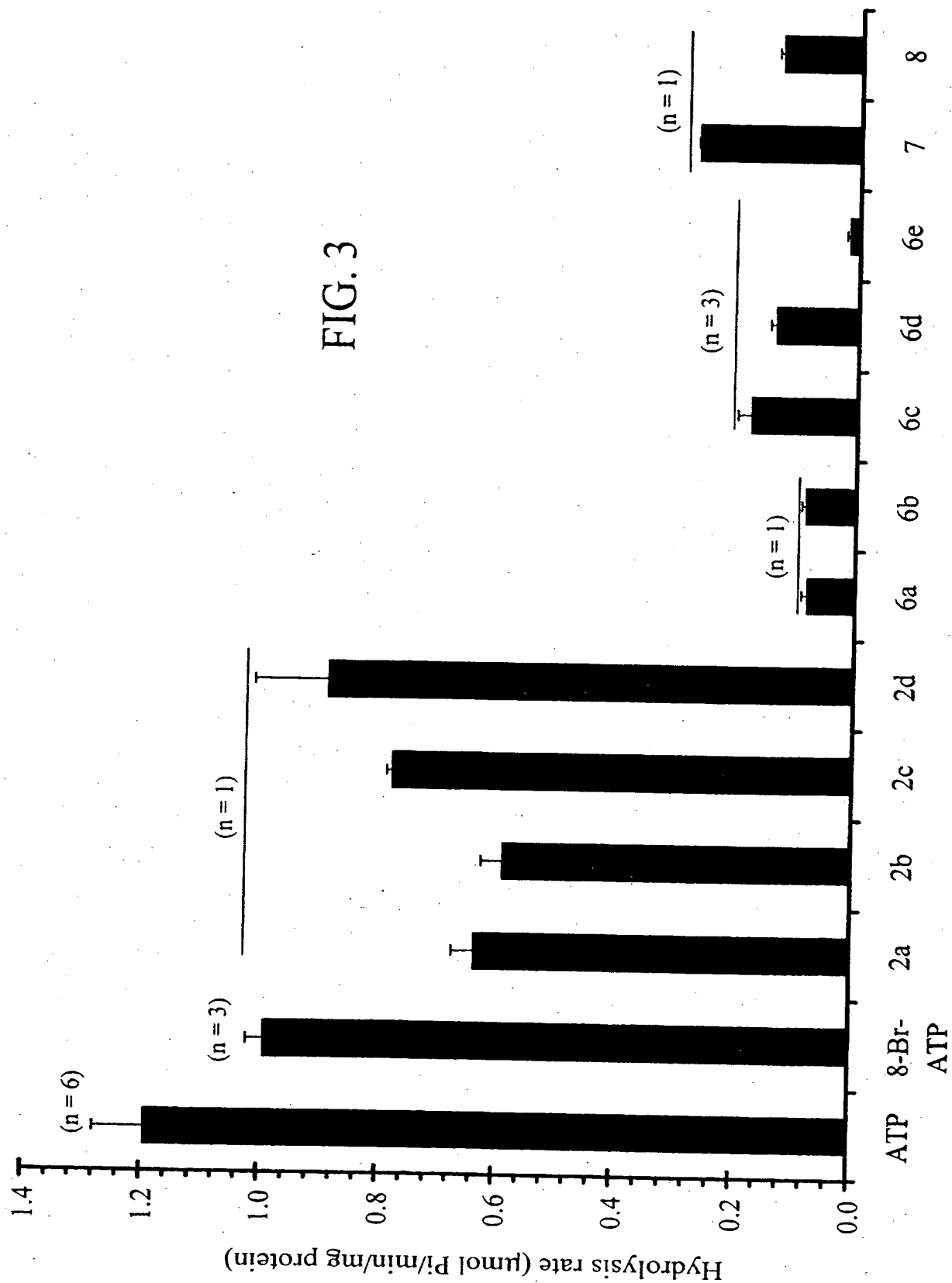


Fig. 2

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FIG. 3



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Substrates	K _m (μ M)	V _{max} (μ mol/min/mg protein)	Inhibitors	K _i (μ M)
ATP	18 \pm 1	1.65 \pm 0.10	8-cycloheptylS-ATP	31 \pm 2.5
ADP	33 \pm 1	1.30 \pm 0.08	8-CH ₂ tBuS-ATP	45 \pm 2.5
2-BuS-ATP 2a	36 \pm 6	0.83 \pm 0.05	8-hexylS-ATP	16 \pm 2.0
2-BuS-ADP 2b	63 \pm 14	0.94 \pm 0.10	8-BuS-ATP	10 \pm 2.0
2-BuNH-ATP 2c	32 \pm 8	0.99 \pm 0.10		
2-BuO-ATP2d	28 \pm 8	0.82 \pm 0.09		
8-bromo-ATP	22 \pm 5	0.63 \pm 0.04		
8-ethylS-ATP 6c	12 \pm 5	0.30 \pm 0.03		
8-BuNH-ATP 7	20 \pm 7	0.28 \pm 0.03		
8-BuO-ATP 8	26 \pm 5	0.20 \pm 0.01		

FIG. 4

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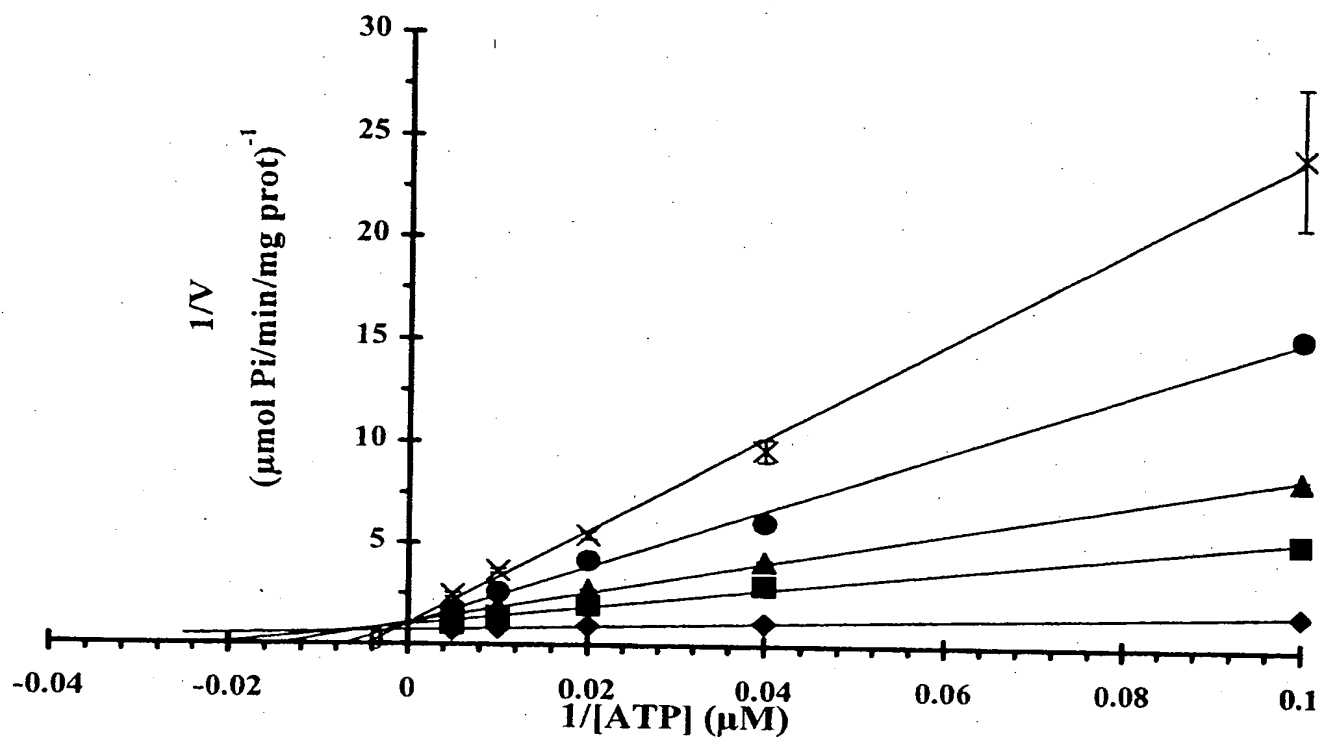


FIG. 5A

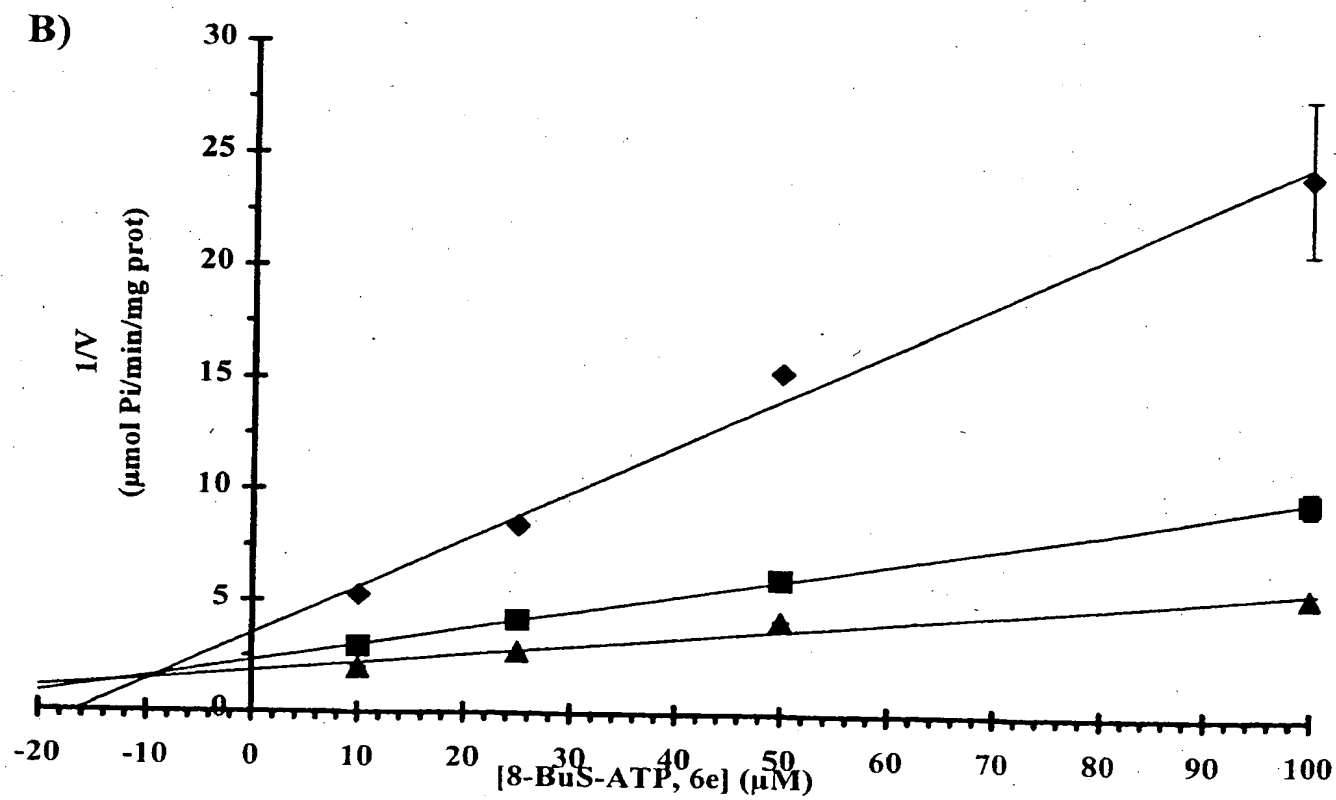


FIG. 5B

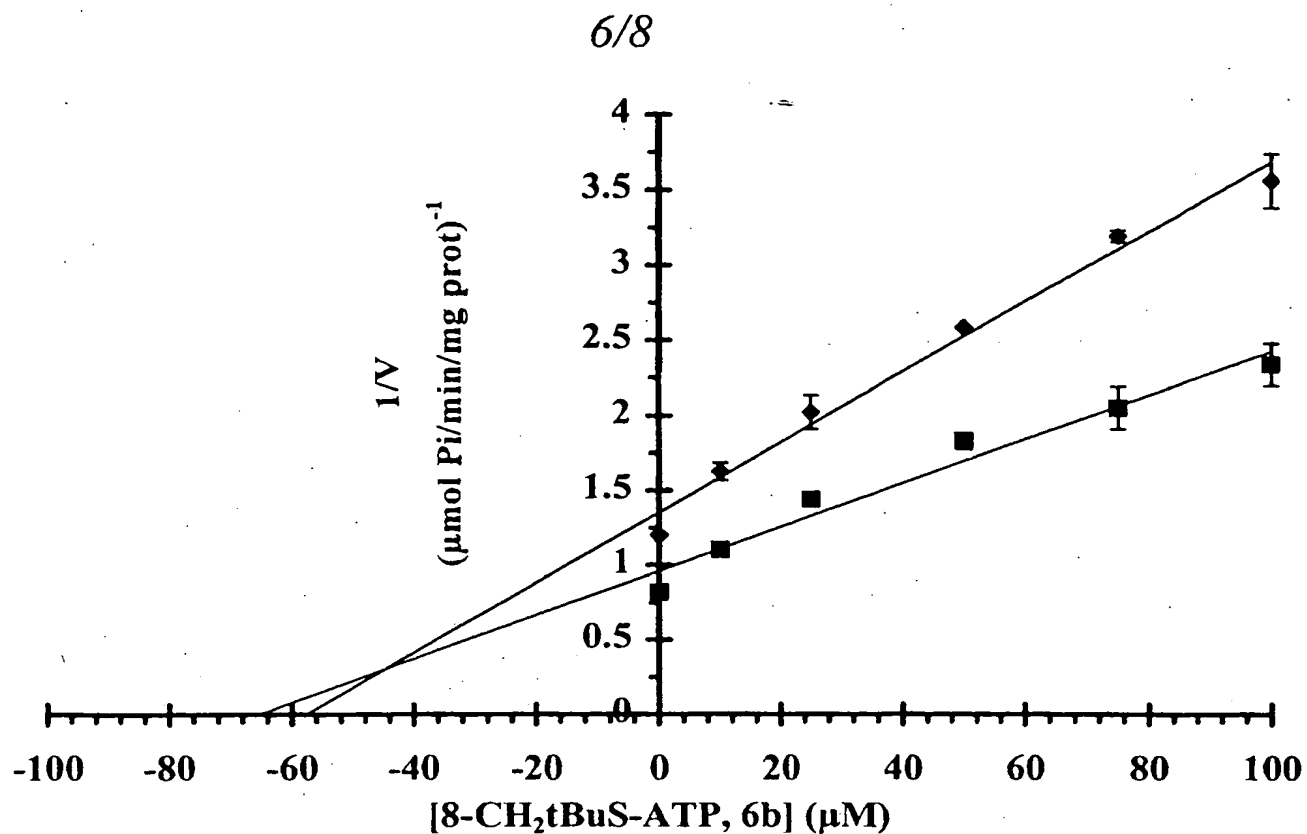


FIG. 6A

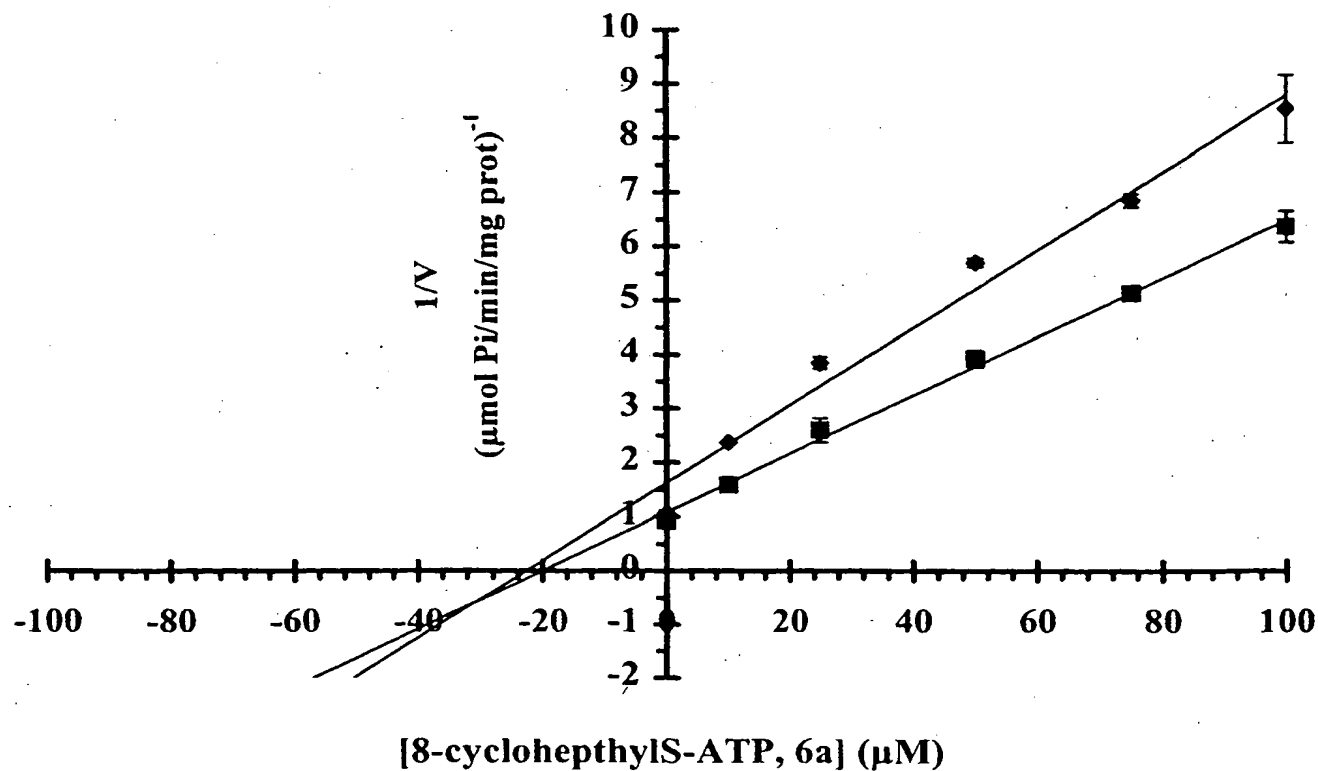


FIG. 6B

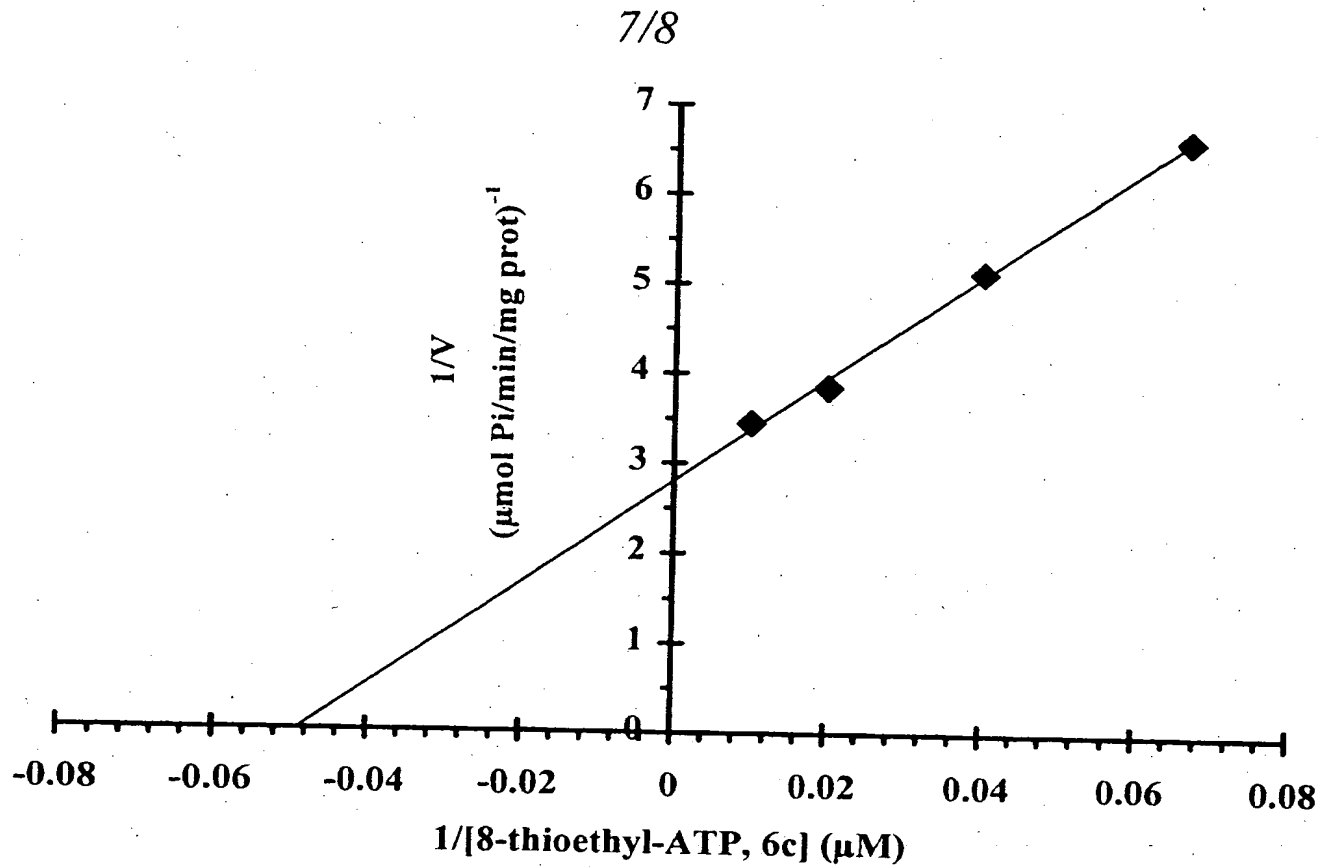


FIG. 7A

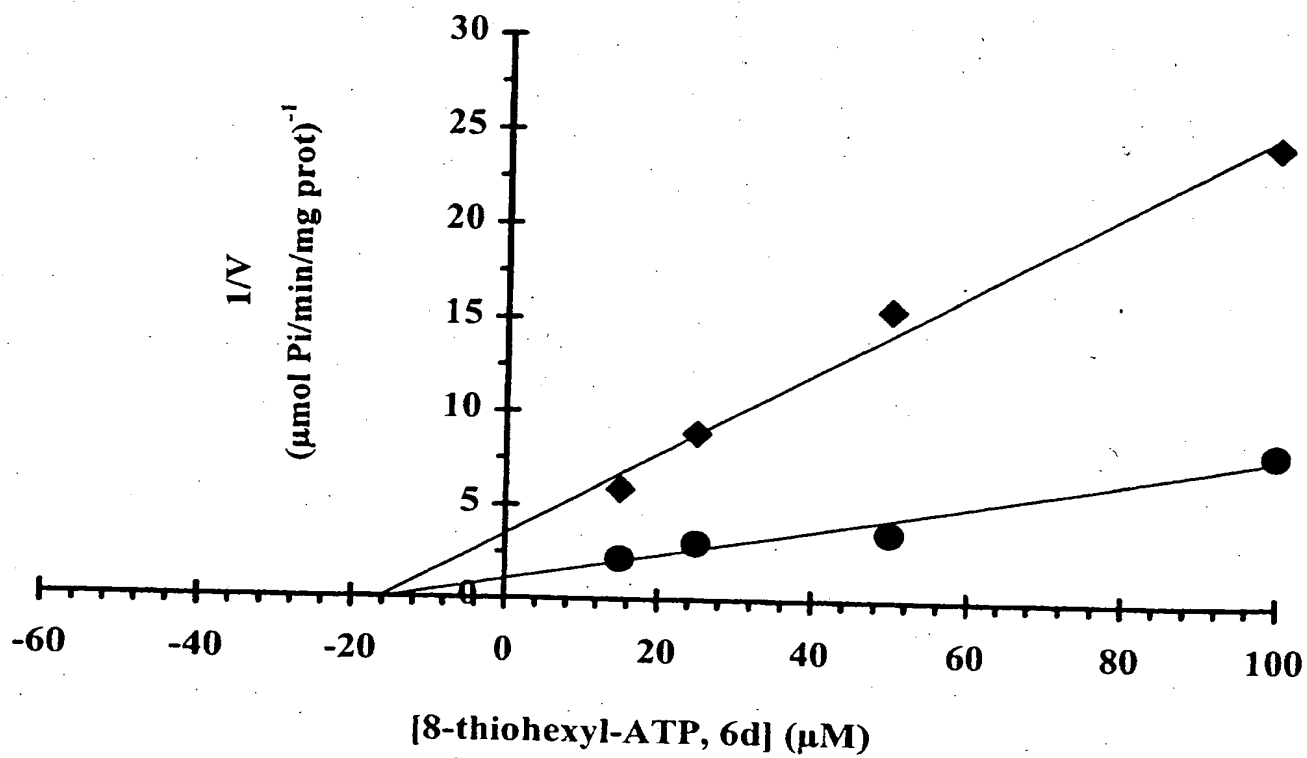


FIG. 7B

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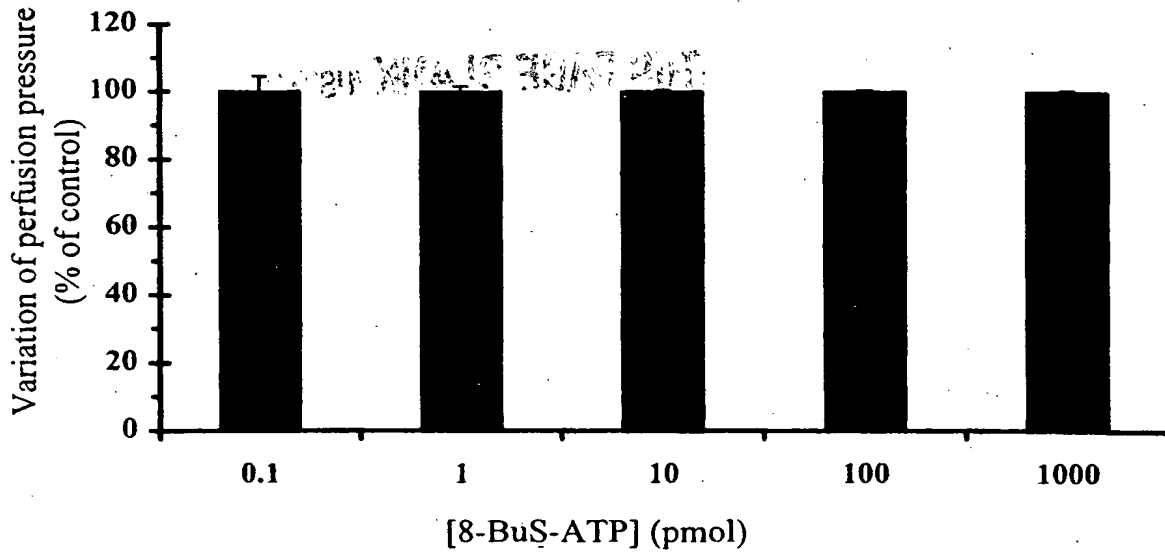


FIG. 8A

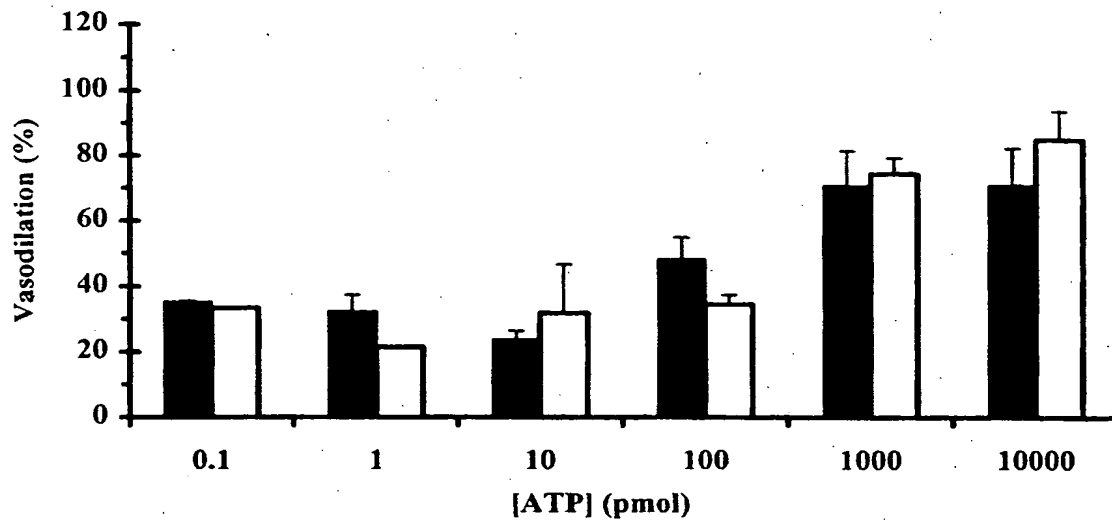


FIG. 8B

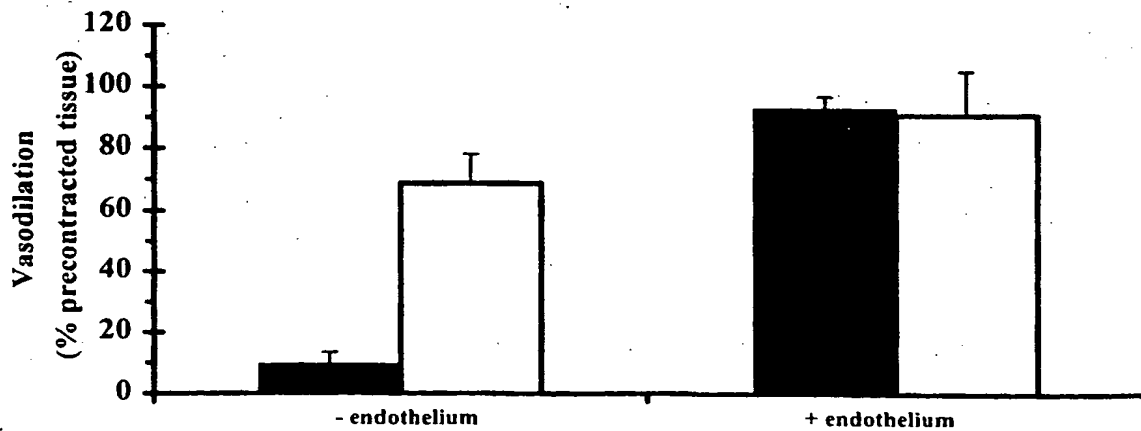


FIG. 8C

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